



Title	Clinical significance of expanded Foxp3+ Helios- regulatory T cells in patients with non-small cell lung cancer(本文)
Author(s)	武藤, 哲史
Citation	
Issue Date	2016-03-24
URL	http://ir.fmu.ac.jp/dspace/handle/123456789/537
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DOI	
Text Version	ETD

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学位論文

**Clinical significance of expanded Foxp3⁺ Helios⁻
regulatory T-cells in patients with non-small-cell
lung cancer**

(非小細胞肺癌患者における Foxp3⁺ Helios⁻ 制御性 T 細胞の増加と,
その臨床的意義)

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Clinical significance of expanded Foxp3⁺ Helios⁺ regulatory T cells in patients with non-small cell lung cancer

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Received July 17, 2015; Accepted September 17, 2015

DOI: 10.3892/ijo.2015.3196

Abstract. The functions of different regulatory T cell (T_{reg}) types in cancer progression are unclear. Recently, expression of the transcription factor Helios was proposed as a marker for natural (non-induced) T_{reg}s. The present study investigated the clinical significance of Helios expression in patients with non-small cell lung cancer (NSCLC). We enrolled 64 patients with NSCLC, of whom 45 were treated surgically and 19 received chemotherapy because of advanced/recurrent disease. Their peripheral blood mononuclear cells were examined by flow cytometry. From the 45 surgery patients, we matched 9 patients with recurrent disease with 9 stage-matched patients without recurrence (n=18), compared their specimens immunohistochemically for tumor infiltrating lymphocytes (TILs) and analyzed these data against clinicopathological factors. Helios expression in Foxp3⁺ T_{reg}s was 47.5±13.3% in peripheral blood and 18.1±13.4% in tumor specimens. Percentage of Helios⁺ T_{reg}s among CD4⁺ T cells were significantly higher in the cancer patients (2.4%), especially those with stage IA disease (2.6%) than in healthy donors (1.5%; P<0.001). Patients with low levels of Helios expression in T_{reg}s among their TILs had significantly poorer survival (P=0.038). Helios⁺ T_{reg}s may affect immune suppression, even in early stage NSCLC; they could also be a useful prognostic biomarker in patients with NSCLC, and possibly a novel cancer immunotherapy target.

Introduction

Lung cancer is the leading cause of cancer death worldwide (1). Although some molecular-targeted drugs provide longer survival time than cytotoxic chemotherapeutic agents (2-6), their efficacy is limited. Earlier cancer immunotherapies

such as several types of vaccines have failed to show clinical effectiveness because the mechanism of immunosuppression has not been fully understood (7). Immune checkpoint inhibitors are widely studied in cancer immunotherapy. These agents, including cytotoxic T lymphocyte-associated antigen (CTLA)-4, PD-1 and PD-L1 inhibitors show 10.2-24% overall response rates in lung cancer patients in early-phase clinical trials (8-12), several agents that target other checkpoint proteins are now in clinical trial pipeline (13). Control of immunosuppression will be an important aspect of effective cancer immunotherapy.

Regulatory T cells (T_{reg}s) have been widely studied in the context of immunosuppression (14). T_{reg}s are characterized by expression of transcription factor Foxp3 (15-17) and are important in the cancer immunosuppressive mechanism. We also reported that T_{reg} levels significantly increase in patients with non-small cell lung cancer (NSCLC), and high peripheral T_{reg} levels correlate with postoperative disease recurrence (18). However, how T_{reg}s expand in cancer and how they influence tumor immunology is unclear.

The T_{reg}s are a heterogeneous population and consist of at least two subsets: natural T_{reg}s (nT_{reg}s) and induced or adaptive T_{reg}s (iT_{reg}s) (19). The nT_{reg}s originate in the thymus and are thought to recognize self-antigens (20). iT_{reg}s develop from conventional naïve T cell precursors at extra-thymic sites by exposure to TGF-β and retinoic acid (21). Because these two T_{reg} types are currently indistinguishable, their relative contributions in tumor immunology are unclear. However, expression of the transcription factor Helios, a member of the *Ikaros* gene family, was recently proposed as a marker for nT_{reg} cells (22). Thereafter, several reports showed iT_{reg}s also express Helios *in vitro* and *in vivo*, which suggests that, rather than a definite marker of nT_{reg}s, Helios could be a marker of T cell activation (23-26). Furthermore, Helios⁺ T_{reg}s reportedly display greater suppressive capacity than do Helios⁺ T_{reg}s (27). Thus, the significance of Helios expression in T_{reg}s is controversial, and its clinical impact in patients with cancer is totally unclear. The percentage of Helios⁺ T_{reg}s was reported as significantly higher in peripheral blood of patients with renal cell carcinoma (RCC) than in healthy donors (HDs) (28). On the other hand, in patients with premalignant respiratory papilloma (PRP), the percentage of Helios⁺ T_{reg}s was significantly reduced among tumor infiltrating lymphocytes (TILs)

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Key words: regulatory T cells, Helios, non-small cell lung cancer, *Ikaros* gene family, Foxp3

Table I. The baseline demographics of the patients.

Characteristics	N=64 (%)
Age (years)	
Mean \pm SD	67 \pm 8
Gender	
Female	27 (42)
Male	37 (58)
Pathological stage	
IA	22 (34)
IB	16 (25)
IIA	4 (6)
IIB	3 (5)
III	0 (0)
IV (recurrence included)	
Chemotherapy	
1st line	6 (9)
2nd line	6 (9)
3rd or later line	7 (11)
Pathological classification	
Adenocarcinoma	51 (80)
Squamous cell carcinoma	10 (16)
Others	3 (5)
EGFR mutation	
Positive	22 (34)
Negative	31 (48)
Unknown	11 (17)

EGFR, epidermal growth factor receptor.

compared with blood (29). However, to the best of our knowledge, this is the first study of Helios⁺ or Helios⁺ T_{reg}s in patients with lung cancer. In trying to understand the clinical influence of Helios expression in patients with NSCLC in the context of other investigations, we found that Helios⁺ T_{reg}s were increased among peripheral T_{reg}s in patients with NSCLC, which implies that Helios⁺ T_{reg}s in the tumor microenvironment might be clinically important in tumor progression.

Materials and methods

Patients. We enrolled 64 patients with non-small cell lung cancer (NSCLC) who were treated at Fukushima Medical University Hospital in 2008, including 45 who were treated by surgery and 19 who were treated by chemotherapy because of advanced or recurrent disease (Table I). Disease staging was evaluated according to the International Staging System for Lung Tumors, 7th edition (30).

Patient specimens

Peripheral blood mononuclear cells (PBMCs). Peripheral blood samples were withdrawn prior to treatment by surgery or chemotherapy. Samples were also taken from 10 HDs. PBMCs were isolated from peripheral venous blood (20–30 ml) using

Ficoll-Paque density gradient centrifugation, and then cryopreserved at -80°C . The PBMCs were thawed for flow cytometry, washed in AIM-V medium (Invitrogen, Carlsbad, CA, USA), counted in the presence of trypan blue dye to evaluate viability and used immediately.

Tumor tissues. Tumor tissues were obtained from patients who i) were suffering from primary NSCLC with confirmed stage (T1–T3, pN0–pN2 and pM0); ii) underwent curative surgery but did not receive any preoperative treatment; and iii) had available clinical follow-up data. All patients had at least 5-year follow-up information for the present study. As 9 patients had recurrent diseases, we selected 9 other recurrence-free patients with matched pathological stages (i.e., 18 patients out of the 45 treated surgically) to examine their immunohistochemical differences in Helios expression in the tumor microenvironment.

Flow cytometry. Cell-surface and intracellular staining procedures were performed as previously described (31). Surfaces of 100 μl of cells (1×10^6) were stained using 10 μl fluorescein isothiocyanate-conjugated anti-CD25 and peridinin chlorophyll-conjugated anti-CD4 (eBiosciences, San Jose, CA, USA). Isotype control, mouse IgG1, was included in all the experiments. For intracellular staining, cells were saponized, washed in cold flow cytometry staining buffer and stained with phycoerythrin-conjugated anti-human Foxp3, its isotype control rat IgG2a (eBiosciences), Adenomatous polyposis coli anti-mouse/human Helios, and its isotype control Armenian Hamster IgG (BioLegend, Inc., San Diego, CA, USA). Flow cytometry was performed using FACSCanto II (BD Biosciences). Acquisition and analysis gates were restricted to the lymphocyte gate, as determined by their characteristic forward and side scatter properties. Flow data were analyzed using FlowJo software, version 7.6.5 (FlowJo, LLC, Ashland, OR, USA).

Immunohistochemistry. We cut 3- μm microtome sections from paraffin-embedded lung cancer specimens and performed immunoperoxidase staining by the avidin-biotin-peroxidase complex method. The sections were dewaxed in xylene and dehydrated through an alcohol gradient. Endogenous peroxidase activity was quenched by 20-min incubation with 0.3% (v/v) solution of hydrogen peroxidase (Wako Pure Chemical Industries Ltd., Osaka, Japan) in 100% methanol. Following incubation in 5% dried skimmed milk in phosphate-buffered saline (PBS) for 30 min at room temperature, the sections were incubated overnight at 4°C with primary monoclonal antibody to Helios (1:50; GTX115629; GeneTex, Inc., Irvine, CA, USA), to Foxp3 protein (1:100; ab20034; Abcam Inc., Tokyo, Japan), CD4 (1:50; NCL-CD4-1F6; Leica Microsystems, Wetzlar, Germany). The primary antibody was then detected using biotinylated secondary anti-rabbit IgG antibody (E0431; Dako, Glostrup, Denmark), or anti-mouse IgG antibody (BA-2000; Vector Laboratories, Burlingame, CA, USA) by the avidin-biotin complex method. The sections were washed several times in PBS after each step and counterstained with Mayer's hematoxylin (Muto Pure Chemicals, Co., Ltd., Tokyo, Japan), dehydrated through an alcohol gradient and mounted on glass slides.

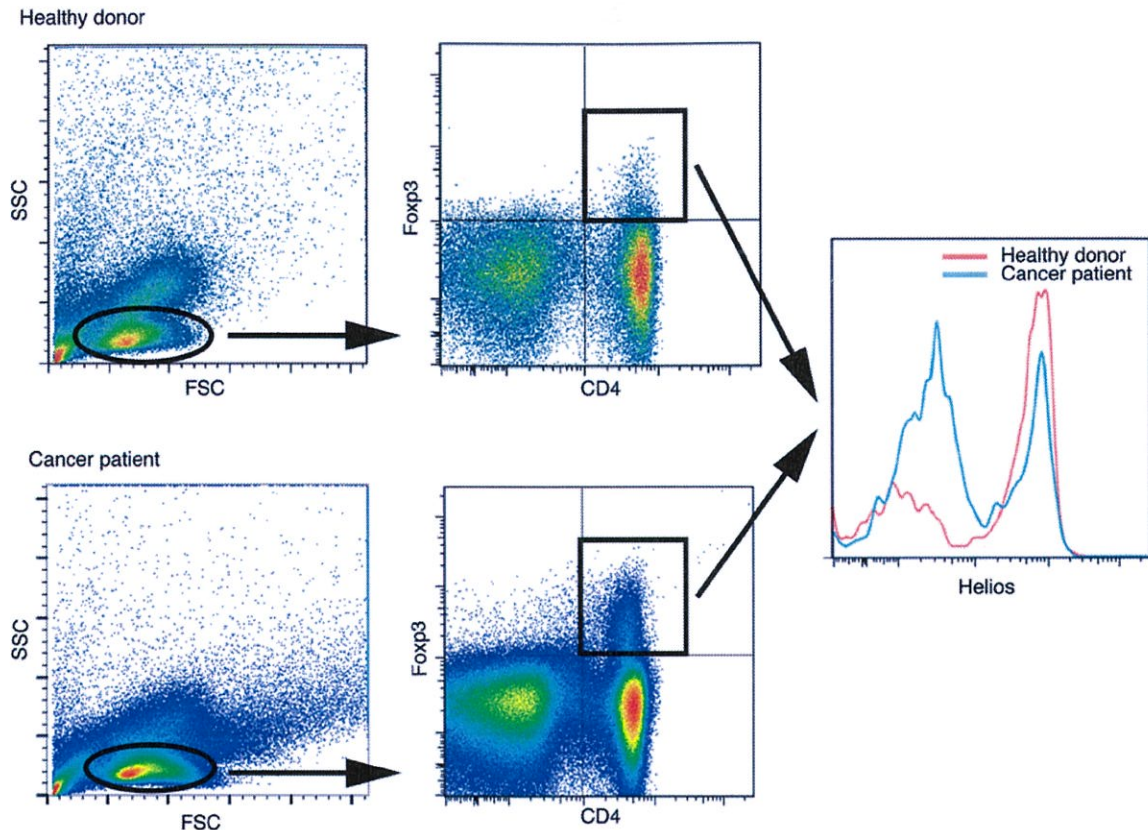


Figure 1. Representative flow cytometry plots of healthy donors and NSCLC patients. NSCLC patients had more Helios⁺ cells among their CD4⁺ Fopx3⁺ T cells than did healthy donors.

For each specimen, we took micrographs of 10 randomly selected fields with a microscope (IX73; Olympus, Co., Tokyo, Japan), a CCD camera (DP73; Olympus), and counted the positively-stained lymphocytes at high-power fields (HPF, x400). We made sure to select the same field for each stain (CD4, Fopx3 and Helios).

Statistical analysis. In peripheral T_{reg}s, first we found percentages of Fopx3⁺ Helios⁺ cells, Fopx3⁺ Helios⁻ cells in CD4⁺ T cells, and Helios⁺ and Helios⁻ cells among CD4⁺ Fopx3⁺ cells. In T_{reg} TILs, we counted the number of CD4⁺ T cells immunohistochemically. Then we counted Fopx3⁺ cells in CD4⁺ T cells, Helios⁺ and Helios⁻ cells in CD4⁺ Fopx3⁺ cells, and analyzed associations between Helios expressions and clinicopathological factors, which were evaluated using Pearson's χ^2 test. Differences between groups were evaluated for statistical significance using the Student's t-test.

Survival curves were drawn according to the Kaplan-Meier method. We compared recurrence-free survival (RFS) and overall survival (OS) between groups of patients who expressed high and low Helios levels among their CD4⁺ Fopx3⁺ cells by log-rank test. All analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered to be statistically significant.

Ethics statement. The present study was approved by the Ethics Committee of the Fukushima Medical University

(no. 2075). Written informed consent was also obtained from all the patients.

Results

Helios expression in peripheral T_{reg}. We first compared the expression of Fopx3 and Helios among CD4⁺ T cells in peripheral blood of 64 patients with NSCLC and 10 HDs. A representative flow cytometry plot of CD4, Fopx3 and Helios expression is shown in Fig. 1. The HDs and NSCLC patients had approximately equal numbers of white blood cells (Fig. 2A) and lymphocytes (Fig. 2B), but the cancer patients had lower CD4⁺ T cell levels ($P = 0.04$, Fig. 2C). Although levels of CD4⁺ Fopx3⁺ T cells (Fig. 2D) did not significantly differ between patients and HDs, the percentage of Fopx3⁺ cells among CD4⁺ T cells was significantly higher in patients (HDs, 3.72%; patients, 5.24%; $P = 0.001$, Fig. 2E). Helios expression in Fopx3⁺ cells was $47.5 \pm 13.3\%$ in patients and $55.9 \pm 12.3\%$ in HDs ($P = 0.07$). Among CD4⁺ Fopx3⁺ T cells, patients had significantly higher Helios⁻ subpopulation levels ($P < 0.0001$) but Helios⁺ cell levels did not significantly differ between HDs and patients (Fig. 3A and B). These results indicate that expanded T_{reg} levels in NSCLC patients are mainly Helios⁻ cells. Notably, even patients with early-stage (stage IA) disease had significantly higher percentages of Helios⁻ T_{reg} among their CD4⁺ T cells than did HDs (HDs, 1.5%; all NSCLC patients, 2.4%; stage IA patients, 2.6%; $P = 0.0005$). The Helios⁻ subpopulation, but not the Helios⁺ subpopulation,

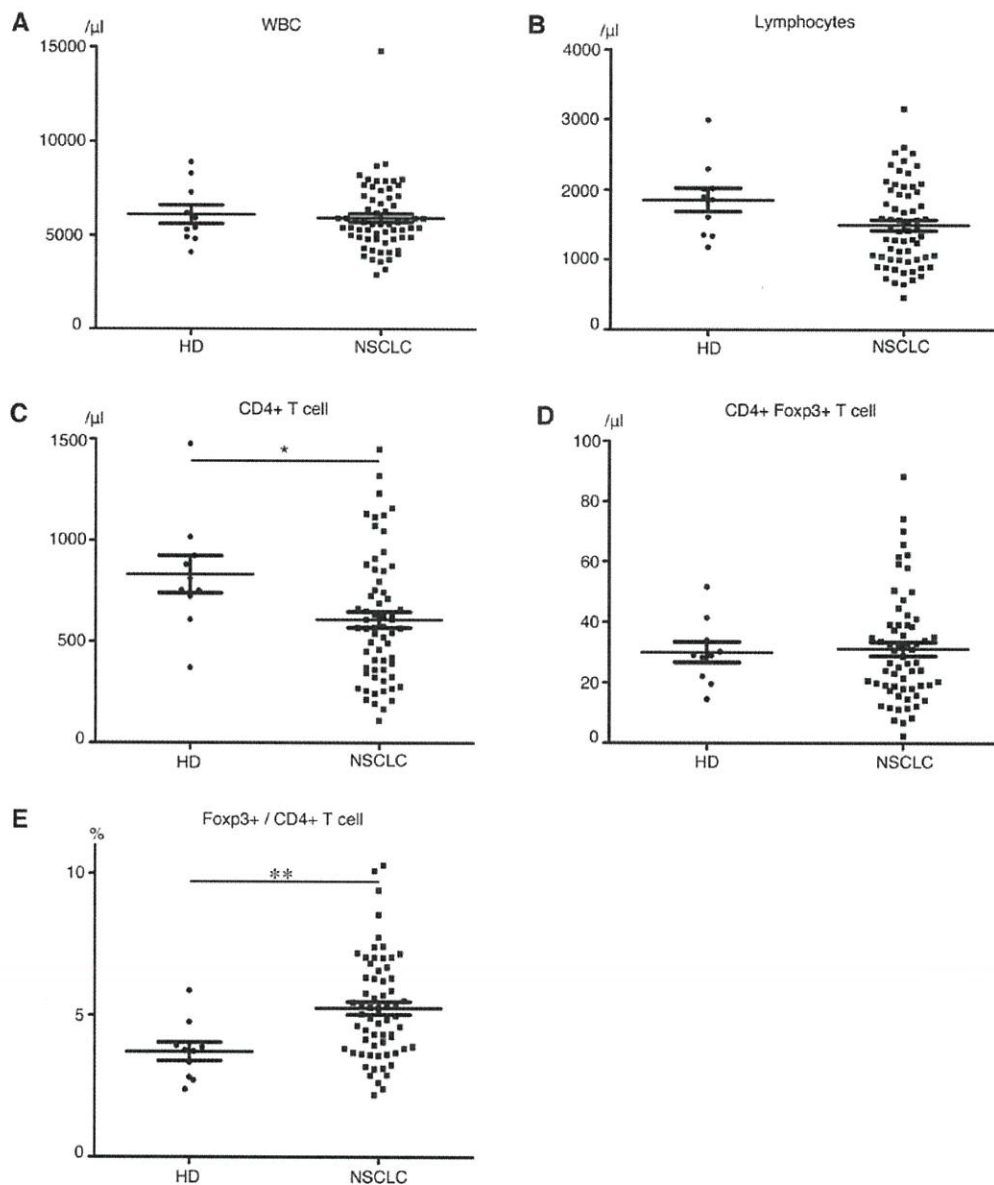


Figure 2. Peripheral blood data. Numbers of (A) white blood cells, (B) lymphocytes, (C) CD4⁺ T cells, (D) CD4⁺ Foxp3⁺ T cells (/mm³). (E) Rates of Foxp3⁺ cells among CD4⁺ T cells (%). Mean \pm SEM. HD, healthy donors; NSCLC, non-small cell lung cancer patients; *P<0.05; **P<0.01.

tended to increase with cancer progression (Fig. 3C and D). We divided patients in high and low Helios-expressing groups by median Helios expression among peripheral CD4⁺ Foxp3⁺ T cells (50.3%), and compared clinicopathological features, but found no significant differences (Table II).

Helios expression in T_{reg} TILs. Next we evaluated T_{reg}s in the tumor microenvironment, and found Foxp3⁺ among CD4⁺ T cells: 18.1 \pm 12.3% (mean \pm SD); Helios⁺ among Foxp3⁺ T cells: 18.1 \pm 13.4%; and Helios⁺ Foxp3⁺ among CD4⁺ T cells: 5.3 \pm 5.2% (Fig. 4). CD4⁺ T cells existed in clusters (Fig. 4A and B), with Foxp3⁺ cells scattered around the CD4⁺ T cells (Fig. 4C). Few Helios⁺ cells were at the same site (Fig. 4D), which indicated that most CD4⁺ Foxp3⁺ cells at the tumor sites were Helios⁻. Thus, the Helios⁺ T_{reg}s percentage was low among both TIL T_{reg}s and peripheral T_{reg}s. We saw a

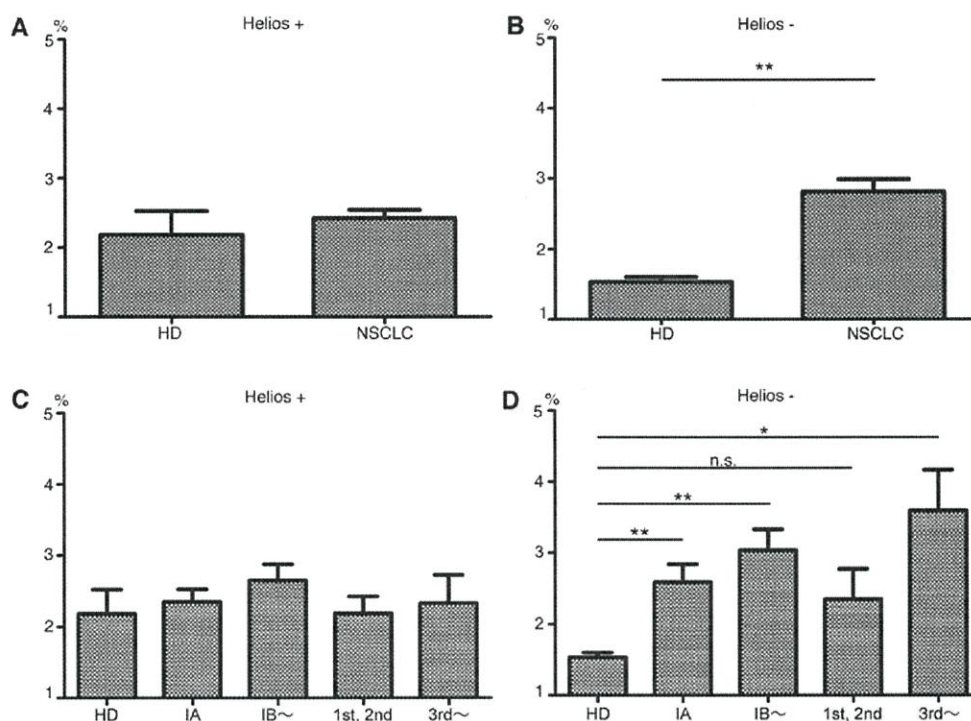
weak but not significant relationship between Helios expression in peripheral-blood T_{reg}s and that in T_{reg} TILs (Fig. 5). Clinicopathological parameters were analyzed against TILs (Table II). When the patients were divided by median Helios⁺ percentage in their TIL CD4⁺ Foxp3⁺ T cells (16.3%) into high- and low-Helios expressing groups, the low Helios expressing group had significantly more advanced-stage disease (P=0.02).

Survival analysis according to Helios expression in T_{reg}. We further analyzed survival outcomes for patients with NSCLC by their Helios expression in T_{reg} among TILs. For the 45 patients who provided PBMCs, median follow-up was 1666 days; relapse occurred in 9 patients and 5 patients died during follow-up. For the 18 patients who provided the specimens for analysis of T_{reg} TILs, the median follow-up was 1525 days; this group included the same patients who relapsed and who died

Table II. Characteristics of patients with NSCLC by levels of Helios expression in their regulatory T cells in PBMCs (N=64) and tumor sites (N=18).

Characteristics	PBMC Helios expression		P-value high vs. low	TIL Helios expression		P-value high vs. low
	High n=32 (50%)	Low n=32 (50%)		High n=9 (50%)	Low n=9 (50%)	
Age (years)						
<65	10 (31)	14 (44)	0.3	4 (44)	1 (11)	0.1
≥65	22 (69)	18 (56)		5 (56)	8 (89)	
Gender						
Female	14 (44)	13 (41)	0.8	6 (67)	4 (44)	0.3
Male	18 (56)	19 (59)		3 (33)	5 (56)	
Pathological stage						
I	18 (56)	20 (63)	0.5	9 (100)	4 (44)	0.02
II	2 (6)	2 (6)		0 (0)	2 (11)	
III	3 (9)	0 (0)		0 (0)	3 (17)	
IV (recurrence included)	9 (28)	10 (31)		0 (0)	0 (0)	
Pathology						
Adenocarcinoma	26 (81)	25 (78)	0.8	7 (78)	9 (100)	0.2
Squamous cell carcinoma	5 (16)	5 (16)		2 (22)	0 (0)	
Others	1 (3)	2 (6)		0 (0)	0 (0)	
EGFR mutation						
Positive	15 (47)	7 (22)	0.1	4 (44)	5 (56)	0.3
Negative	13 (41)	18 (56)		3 (33)	4 (44)	
Unknown	4 (13)	7 (22)		2 (22)	0 (0)	

PBMC, peripheral blood mononuclear cell; TIL, tumor infiltrating lymphocyte; EGFR, epidermal growth factor receptor.

Figure 3. Rates of Helios⁺ Foxp3⁺, Helios⁻ Foxp3⁺ cells among CD4⁺ T cells of HD and NSCLC. Data of HD and NSCLC patients in panels (A and B) were divided into four groups: preoperative stage IA; preoperative stage ≥IB; 1st or 2nd line chemotherapy; and ≥3rd line or more chemotherapy for recurrence in panels (C and D), respectively. Mean ± SEM. HD, healthy donors; NSCLC, non-small cell lung cancer patients; *P<0.05; **P<0.01.

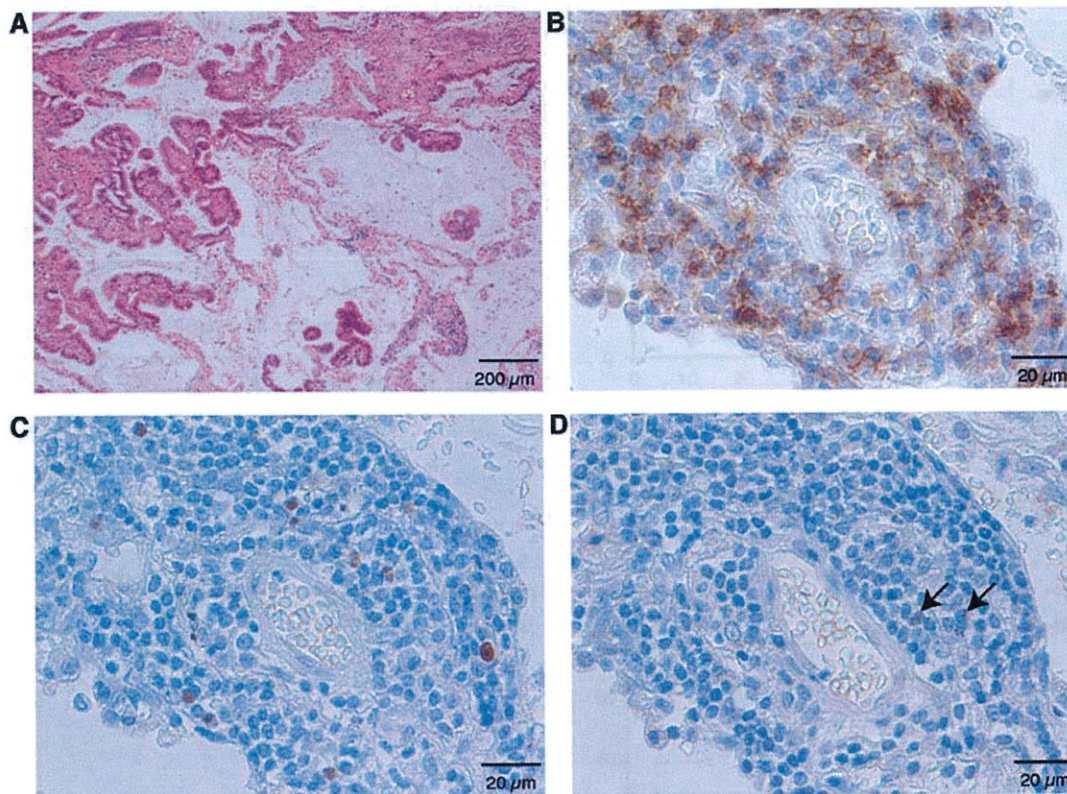


Figure 4. Representative micrographs of tumor tissue. Stained by (A) H&E; (B) anti-CD4 antibody; (C) anti-Foxp3 antibody; and (D) anti-Helios antibody in the same section of tumor tissue. Black arrow, positive cells (D).

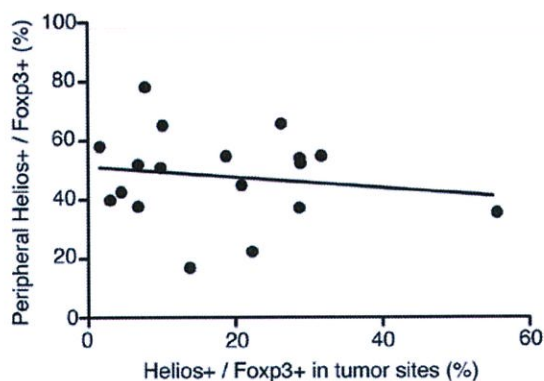


Figure 5. The relationship between Helios expression in peripheral blood T_{reg}s and that in tumor site. They were weakly correlated. $R^2=0.03$.

in the former group. The patients who provided tumor specimens did not significantly differ in RFS or OS from those who provided only PBMCs. Patients with lower Helios expression among their T_{reg} TILs had significantly poorer OS ($P=0.038$), but not significantly poorer RFS (Fig. 6).

Discussion

To date, very few reports have addressed Helios expression in peripheral CD4⁺ Foxp3⁺ T cells. According to these previous reports, the percentage of Helios⁺ T_{reg}s in patients with RCC was ~60% (28) and in PRP was $66.1 \pm 6.5\%$ (29). In the

present study, we show that Helios expression among CD4⁺ Foxp3⁺ cells was $47.5 \pm 13.3\%$ in patients with NSCLC, lower than previous data but almost compatible. The percentage of Helios⁺ among CD4⁺ Foxp3⁺ T cells was significantly lower in these patients than those of HDs (NSCLC, 47.5%; HD, 55.9%). This is the first report of Helios expression status in T_{reg}s from patients with NSCLC; it was significantly decreased in patients with NSCLC than in HDs. Interestingly, even higher percentages of Helios⁺ T_{reg}s were seen in advanced-stage NSCLC. These results show expanded T_{reg}s in patients with NSCLC to be Helios⁺ cells, which implies that Helios⁺ T_{reg}s mediate immunosuppression in NSCLC. These results are inconsistent with the earlier reports on RCC and PRP, and suggest that the role of these cells in immunosuppression may vary between the type of tumor. However, these data are still limited; further studies are needed to understand these findings.

Among TILs, Helios expression in Foxp3⁺ cells was $18.1 \pm 13.4\%$ in patients with NSCLC in this series. Helios⁺ T_{reg}s in tumor sites were associated with patient prognoses. Wainwright *et al* reported that Helios was expressed by almost 90% of T_{reg}s in glioblastoma (32), but in only $31.2 \pm 18.51\%$ of T_{reg}s in PRP (29). These data also imply that the immunosuppressive status in the tumor microenvironment may depend on the type of tumor. Our findings indicate that Helios⁺ T_{reg}s have an essential role in immunosuppression, at least in NSCLC. According to the previous study showing that Helios could be a biomarker for nT_{reg}s (22), the present study suggests that iT_{reg}s were mainly increased in patients with NSCLC patients and might be induced in tumor microenvironment.

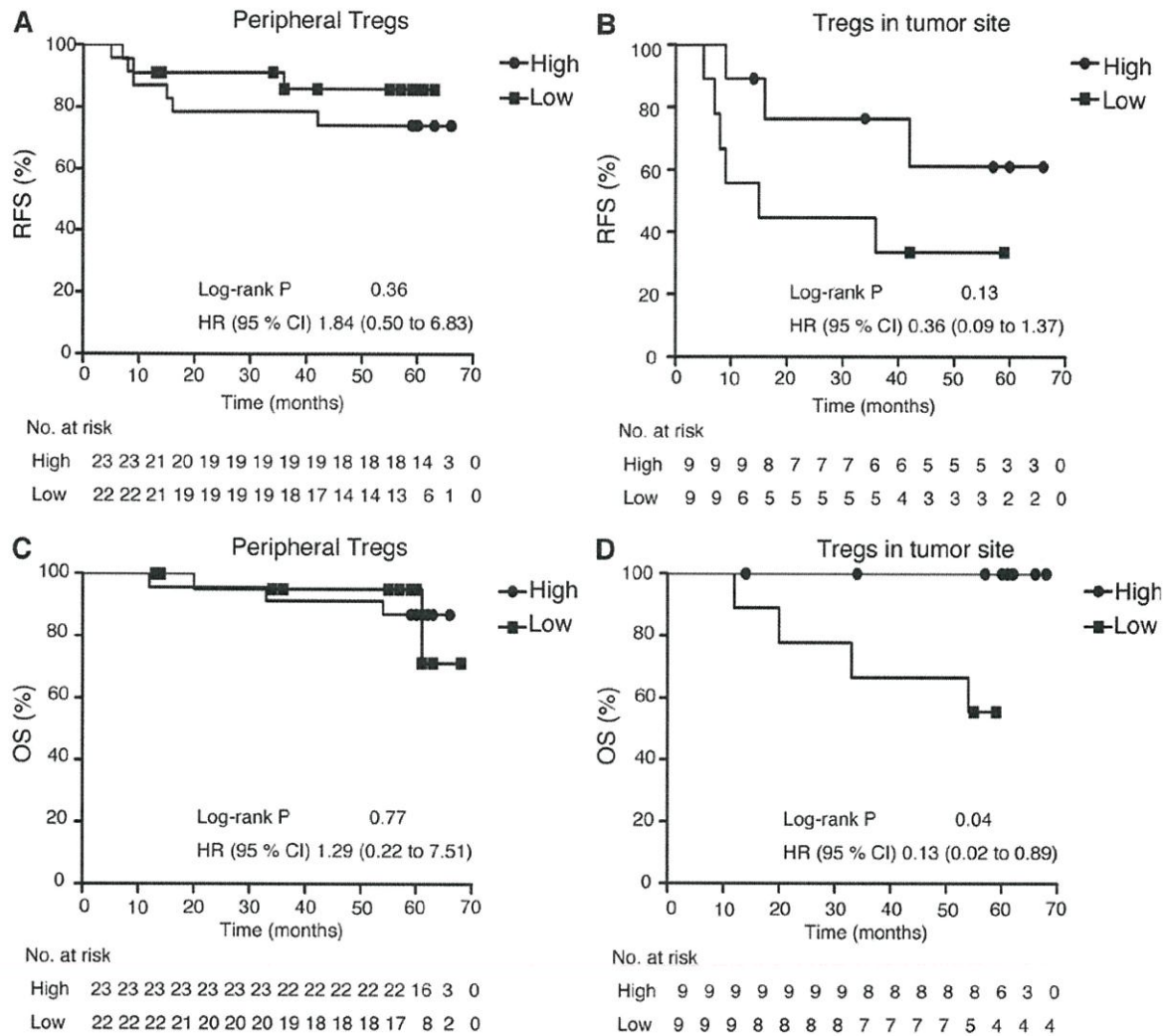


Figure 6. Survival curve of patients who expressed high or low levels of Helios in CD4⁺ Foxp3⁺ T cells. (A) Recurrence-free survival (RFS) and (B) overall survival (OS) by peripheral CD4⁺ Foxp3⁺ T cells. (C) RFS and (D) OS by CD4⁺ Foxp3⁺ T cells in tumor sites.

However, recent reports suggest that nT_{reg}s from iT_{reg}s are not distinguished only by Helios expression (25). Other molecular markers are now under study. Several promising molecules are epigenetic T_{reg}-specific demethylated region (TSDR) modifications and Neuropilin 1. Reportedly, epigenetic modifications in the TSDR of the *Foxp3* locus affect the stability of Foxp3 (33), and are thought to differ between nT_{reg}s from iT_{reg}s cells (34). Neuropilin 1 is a receptor for vascular endothelial growth factor and semaphorin family proteins (35), and is reported to be a possible marker for nT_{reg}s (36,37). In spite of these studies, a definitive marker for nT_{reg}s has not been found so far (38).

Our findings suggest that Helios⁺ T_{reg}s could have an important function in NSCLC. We must next elucidate the function of Helios⁺ T_{reg}s in immunosuppression to verify their role in cancer immune response. For such a study, we need to obtain a definitive cell-surface marker for Helios⁺ T_{reg}s, as Helios is a transcription factor. To solve this problem, Neuropilin 1 may be useful as mentioned above (35). Other cell-surface markers have been reported. Zabransky *et al* reported CD103 and glucocorticoid-induced tumor necrosis receptor (GITR) (25), and Raffin *et al* described IL-1RI and CCR7 (27). Currently,

we are trying to separate Helios⁺ T_{reg}s from other CD4⁺ T cells using IL-1RI and CCR7, which gave 70-80% specificity (data not shown). Further studies are needed to find a definitive cell-surface marker and establish the effects of Helios⁺ T_{reg}s on immunosuppression.

Successful blockades of immune checkpoints suggest that immune escape mechanisms clearly contribute to lung cancer progression, and also could be targets for lung cancer treatment. To date, attempts have been made to suppress T_{reg}s function by using anti-CD25 antibody (39-41), and by inhibiting the CTLA-4 pathway (12,42), or GITR family-related proteins (43). These trials did not work well. According to the present study, Helios⁺ T_{reg}s could be both a useful prognostic biomarker in NSCLC patients and a therapeutic target. If we can ascertain the mechanism of immunosuppression, we may be able to establish more powerful immunotherapy. For instance, peptide vaccine has been one of the main streams of immunotherapy. Although peptide vaccine therapy for NSCLC could not achieve a survival benefit in a late-phase studies (44), other trials, such as our multiple peptide vaccine therapy for NSCLC, have been expected to induce strong specific T cell

responses (45). Combination therapy of blockades of immunosuppressive pathway, including immune checkpoints and/or T_{reg}s that target Helios⁺ T_{reg}s and these peptide vaccines could be a next-generation immunotherapy.

In summary, in the present study we demonstrated the clinical impact of both local and systemic Helios⁺ T_{reg} cells. Systemic Helios⁺ T_{reg}s correlate with advanced cancer stage, and those in tumor sites are associated with poor patient prognosis. Thus, analyzing Helios expression levels in T_{reg}s could be a useful marker for disease progression and prognosis in patients with NSCLC. Furthermore, it also could be a novel therapeutic target for various cancers. Further study is needed to understand the function of Helios⁺ T_{reg}s in immune suppression, and to develop therapeutic modality targeting cancer-specific T_{reg}s.

Acknowledgements

We thank E. Ohtomo, Y. Kikuta and Y. Yuda for their excellent technical assistance.

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